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CHEMICAL CONSTRUCTS AND THEIR USES

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5 Field of the Invention

This invention relates to chemical constructs for use in solid phase synthesis, and to methods of detecting and/or characterising the products of solid phase synthesis using the constructs.

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Background of the Invention

Solid phase synthesis has been known for many years in the field of peptide synthesis and more recently has also been used increasingly for the synthesis of non-peptides.

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Solid phase synthesis has found particular application in the field of combinatorial chemistry and the preparation of chemical libraries as potential sources of new leads for drug discovery, see for example Anthony W. Czarnik, *Analytical Chemistry News and Features*, June 1, 1998, pp 378A-386A, and *The Combinatorial Index*, Barry A. Bunin, Academic Press, San Diego 1998. A feature of combinatorial chemistry methods is that they enable very large numbers of different compounds to be prepared from a relatively limited number of molecular building blocks in a relatively small number of reactions. Combinatorial chemistry makes use of the "split and pool" approach in which a suspension of chemical starting material tethered to a solid support is split into N portions, each of which is reacted with a different reagent. The products of the N reactions are then pooled and mixed thoroughly, the resulting pool is split into N' portions and again each portion is reacted with a different reagent. This procedure can be repeated as many times as there are steps in the reaction sequence. Thus, for a three step reaction sequence, if the reaction mixture is divided into ten portions at each stage, each of the pools being reacted with a different reagent before recombining with the other portions, the total number of compounds formed by the process will be  $10^3 = 1000$ . Thus it can be seen that by using the split and pool technique, a large number of different molecules can be synthesised using a minimal number of reactions (thirty in the case mentioned above).

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Each of the solid supports (e.g. a resin bead) will have a single product molecule

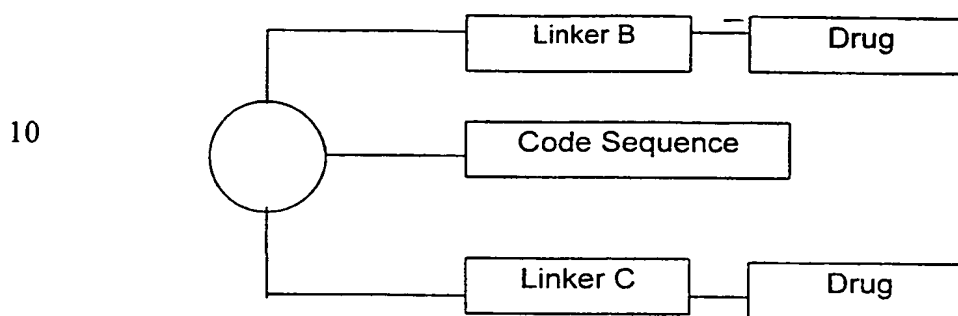
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tethered to it and hence in principle each of the reaction products can be separated and analysed or subjected to biological testing simply by isolating each single solid support and cleaving the product from the support. However, the numbers of compounds generated in large libraries by combinatorial methods means that it can be impracticable to identify and characterise each compound. Consequently, the compounds usually are first tested, either on the solid support or after cleaving from the support, and only those compounds which show some biological activity are subsequently identified. In order to minimise the number of biological tests carried out, compounds can be tested in pools containing a predetermined number of compounds, the inactive pools being discarded and the active pools being subject to further investigation. The biological activities of the compounds can be analysed using high throughput automated assay techniques permitting large numbers of compounds to be analysed in a short time.

One of the problems facing the chemist is how to identify and characterise the various compounds in a combinatorial library since each compound will be present in the library only at very low concentrations, and there will usually be insufficient compound present on a given solid support to allow for both biological testing and identification of the compound. This problem has been addressed by providing each solid support with a coding tag from which the identity of the compound can be determined. Thus, for example, a coding tag can be built up in sequential steps on the solid support in parallel with the construction of the desired target compound, the coding tag reflecting the synthesis history of the product compound and being unique for each product compound. The coding tag is usually built up on the support using chemical reactions of a type which are orthogonal to the chemistry used to build up the product compound, thereby ensuring that the coding units and product compounds do not become confused. Once the product compound has been tested, and its biological activity confirmed, the coding tag can then be decoded to allow identification of the compound.

Where a product compound has been subjected to a preliminary biological screen as part of a pool of compounds, and the pool has been shown to have activity, it is then necessary either to look at smaller pools of compounds or individual compounds in order to discover the identity of the active compound. In many assay systems, it is necessary to cleave the compound from the solid support in order to carry out biological testing, and this can lead to problems with the pool testing approach. Thus, if all of the active compound has been cleaved from the solid support in order to carry out the testing, such that the pool contains a mixture of a large number of dissolved compounds, recovery of each individual compound

for further testing would create a severe separation problem. In order to overcome or avoid this problem, "differential release" constructs have been created in which the product compound, e.g. a drug molecule, is linked to the solid support by several different types of linker groups, each of the linker groups being orthogonally and selectively cleavable under different chemical conditions. Such a construct (Construct A) is shown schematically below.



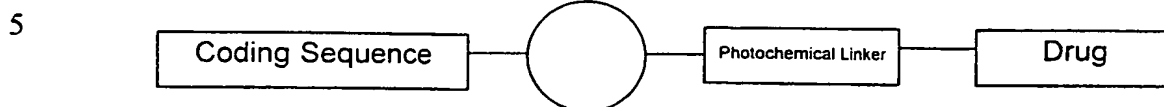
**Construct A**

Following a combinatorial synthesis scheme, the reaction products can be divided into x pools each containing y solid supports, each solid support bearing a different product drug molecule. Each pool can then be subjected to cleavage conditions suitable for selectively cleaving linker B so as to free a proportion of the total drug present on the solid support. The solid supports can then be removed and the residual solution subjected to the desired biological assays. If the pool as a whole shows biological activity, the individual partially cleaved solid supports in that active pool can then be separated into individual containers and subjected to conditions effective to bring about cleavage of the second linker, linker C. Subsequently, the individual drugs can each be screened for biological activity.

Once biological activity has been observed for drug associated with a particular individual solid support, the support is subjected to conditions which allow the tagging code to be decoded and the identity of the drug compound established.

An alternative to "differential release" is "tiered release". In a tiered release system, the construct does not employ two orthogonal cleavage sites as with the differential release constructs, but makes use of only a single cleavage site. A proportion of the drug or test compound is released in a first cleavage step to provide sufficient compound for a first biological assay, and subsequently more drug can be released by cleavage at the same cleavage site for further biological assays. Such tiered release systems make use of cleavage

reactions which are relatively slow, and where the rate of cleavage can be reasonably well controlled. Photochemical cleavage is typically used in tiered release systems. A construct for use in a tiered release system is shown below as Construct B.



**Construct B**

10 In any solid phase synthesis procedure, whether or not part of a combinatorial procedure, it is important to be able to determine the optimal conditions for a given reaction step in a series of steps. It is also important to be able to monitor the progress or path of a reaction so that it can be determined whether a particular reaction has gone to completion, or indeed whether a particular reaction has taken place at all. This is particularly important in a multistage solid phase synthesis where the failure of a given stage to proceed to completion  
15 can lead to the formation of side products thereby complicating what is otherwise a relatively straightforward separation procedure.

20 Although both invasive and non-invasive analytical methods are known for determining the products of solid phase synthesis (see *The Combinatorial Index, idem*), one of the recognised problems with solid phase synthesis is that it is generally more difficult to monitor the progress of a reaction; see for example the Czarnik paper referred to above. This is particularly true with the products of combinatorial chemistry methods where there is a need to use rapid high throughput techniques to analyse the large numbers of compounds generated by such methods. Techniques such as mass spectrometry are potentially ideally  
25 suited as a means of providing high throughput analyses, but a substantial problem is that not all compounds produce a guaranteed response under mass spectrometric conditions. Indeed, many compounds are "invisible" to the so-called "soft" methods of mass spectrometry such as Matrix-Assisted Laser-Desorption Ionisation (MALDI) and Electrospray mass spectrometry which are intended to detect molecular ions without causing significant fragmentation of the  
30 molecule. One of the reasons for this is that under MALDI and electrospray conditions, many compounds, particularly peptides, do not ionise sufficiently well to give a detectable mass spectral response.

35 It is an object of the present invention to provide a means of monitoring the progress or path of a chemical reaction on a solid support, for example in a combinatorial synthesis,

which avoids problems inherent in known methods and which provides a means of detecting and identifying the products of solid phase synthetic techniques using mass spectroscopy.

Accordingly, in a first aspect, the invention provides a chemical construct for use in solid phase synthesis comprising a solid support Q having linked thereto groups Y<sup>1</sup>R and Y<sup>2</sup>R; wherein R is a substrate or a coding tag and the groups Y<sup>1</sup> and Y<sup>2</sup> are connecting groups each having a first cleavage site, at least one of Y<sup>1</sup> and Y<sup>2</sup> having a second cleavage site located between the first cleavage site and group R, the first cleavage site being orthogonally and selectively cleavable with respect to the second cleavage site, and, when both groups Y<sup>1</sup> and Y<sup>2</sup> contain a second cleavage site, the second cleavage site in Y<sup>1</sup> being selectively and orthogonally cleavable with respect to the second cleavage site in Y<sup>2</sup>; the second cleavage site being cleavable to release the substrate; and the first cleavage site being selectively cleavable to release a fragment Fr comprising the substrate R and at least a portion of the connecting group Y; and wherein:

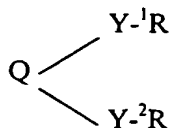
- (i) the chemical fragment Fr contains a sensitising group G which sensitises the chemical fragment Fr to instrumental, e.g. mass spectroscopic analysis and/or;
- (ii) the fragment Fr contains a means for imparting a characteristic signature to the mass spectrum of the fragment.

Preferably at least one group R is a substrate and more preferably the substrate is a drug molecule or other molecule having biological activity.

In one preferred embodiment, there is provided a chemical construct for use in solid phase synthesis comprising a solid support Q having linked thereto groups Y<sup>1</sup>R and Y<sup>2</sup>R; wherein R is a substrate (such as a drug molecule) and the groups Y<sup>1</sup> and Y<sup>2</sup> are connecting groups each having first and second cleavage sites which are orthogonally and selectively cleavable, the second cleavage site in Y<sup>1</sup> being selectively and orthogonally cleavable with respect to the second cleavage site in Y<sup>2</sup>; the second cleavage site being cleavable to release the substrate; and the first cleavage site being located at a position between the second cleavage site and the solid support and being selectively cleavable to release a fragment Fr comprising the substrate R and at least a portion of the connecting group Y; and wherein:

- (i) the chemical fragment Fr contains a sensitising group G which sensitises the chemical fragment Fr to instrumental, e.g. mass spectroscopic analysis and/or;
- (ii) the fragment Fr contains a means for imparting a characteristic signature to the mass spectrum of the fragment.

The constructs of the invention can be illustrated by the general formula:



5        The constructs of the invention provide a differential release system which is more readily analysable to determine the outcome of individual reactions on the solid support. Thus, for example, by subjecting the construct to cleavage conditions effective selectively to bring about cleavage at each of the first cleavage sites, chemical fragments containing the substrates R linked to an "analytical lever" are produced in which the detectability of the chemical fragments, and hence the substrates, is enhanced. Thus, cleavage at the first  
10        cleavage sites can be used to determine whether a particular substrate has been formed, a facility which is useful as a means of carrying out quality control (QC) during a reaction scheme or library generation, and which provides a means of analysis that can be used when optimising the conditions for a given synthetic process step.

15        The constructs can contain further groups  $\text{Y}^n$ , each of which has a second cleavage site which is selectively and orthogonally cleavable with respect to the second cleavage sites in the other Y groups. Thus, for example, the constructs can contain a group  $\text{Y}^3\text{Q}$  in which the second cleavage site in the group  $\text{Y}^3$  is cleaved under different chemical conditions from  
20        the second cleavage sites in  $\text{Y}^1\text{R}$  and  $\text{Y}^2\text{R}$ . Thus by means of the invention, it is possible to provide differential release of product substrate molecules (e.g. drugs) in two, three, four or more steps, thereby assisting the products of large combinatorial libraries to be screened biologically in pools of manageable size.

25        In a second aspect, the invention provides a differential release method of assaying a chemical library for biological activity, the method comprising:

- (i) subjecting a construct comprising a solid support Q having linked thereto groups  $\text{Y}^1\text{R}$  and  $\text{Y}^2\text{R}$  as hereinbefore defined to cleavage conditions effective to release substrate R from the group  $\text{Y}^1\text{R}$ ;
- 30        (ii) testing the substrate R released from the group  $\text{Y}^1\text{R}$  in a biological assay;
- (iii) subsequently subjecting the construct to cleavage conditions effective to release substrate R from the group  $\text{Y}^2\text{R}$ ; and
- (iv) ) testing the substrate R released from the group  $\text{Y}^2\text{R}$  in a biological assay.

35        In addition to providing a differential release method of analysing a library, the

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invention also provides a method of tiered release analysis which makes use of constructs which are selectively cleavable to release a fragment Fr comprising the substrate R and at least a portion of a connecting group Y; and wherein:

- 5 (i) the chemical fragment Fr contains a sensitising group G which sensitises the chemical fragment Fr to instrumental, e.g. mass spectroscopic analysis and/or:
- (ii) the fragment Fr contains a means for imparting a characteristic signature to the mass spectrum of the fragment.

Accordingly, in a further aspect, the invention provides a tiered release method of assaying a chemical library for biological activity, the method comprising:

- 10 (i) subjecting a construct comprising a solid support Q having linked thereto a group Y<sup>1</sup>R as hereinbefore defined to cleavage conditions effective to release a first portion of the substrate R from the group Y<sup>1</sup>R;
- (ii) testing the first portion of substrate R released from the group Y<sup>1</sup>R in a biological assay;
- 15 (iii) subjecting the construct to cleavage conditions effective to release a second portion of the substrate R from the group Y<sup>1</sup>R; and
- (iv) testing the second portion of substrate R released from the group Y<sup>1</sup>R in a biological assay.

20 In each of the above differential release and tiered release methods of the invention, the cleavage step (i) is typically performed on a pool of solid supports, each bearing a different substrate R and, following the cleavage step (I), the solid support is typically separated from the released substrate R. If biological activity is detected in the pool, the solid supports can be isolated, or grouped into smaller pools, and then subjected to cleavage step (iii) to release further substrate for testing so that the biological activity can be traced to a particular solid support or small group of supports.

To assist in determining the identities of the products of a combinatorial synthesis scheme, each solid support can contain a coding tag or coding sequence which encodes information indicative of at least part of the synthesis history of the construct. The coding tag can be built into the solid support itself or a coding sequence can be linked to the solid support. The coding sequence preferably is linked to the solid support by means of a connecting group Y<sup>a</sup> having a cleavage site cleavable to release a fragment F<sup>a</sup> from the solid support, the fragment F<sup>a</sup> comprising the coding sequence and optionally at least a portion of

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the connecting group  $Y^a$ .

It is preferred that:

5 (i) the chemical fragment  $F^a$  contains a sensitising group G which sensitises the chemical fragment  $F^a$  to instrumental, e.g. mass spectroscopic analysis and/or:

(ii) the fragment  $F^a$  contains a means for imparting a characteristic signature to the mass spectrum of the fragment.

10 The coding tag can take the form of a group or sequence of coding groups that can be fragmented, and in particularly sequentially fragmented, under mass spectrometric conditions to provide one or more characteristic mass spectral peaks that provide a code from which the substrate can be identified. For example, the coding tag can comprise a sequence of different amino acids, e.g. two, three or four amino acids, the identity and order of the amino acids forming a code indicative of the identity of the substrate. Under mass spectrometry  
15 conditions, the coding groups (e.g. amino acids) can be sequentially cleaved to give a series of characteristic mass spectral fragments, relating to coding tags in which, for example, one or two or more amino acids have been lost from the tag. By relating the masses of the fragment peaks back to the mass of the parent coding tag, it is possible to identify the individual coding groups (e.g. amino acids) and their position on the coding tag, thereby enabling the code to be  
20 broken and the substrate identified. By providing a sensitising group G and a means for imparting a characteristic signature to the mass spectrum, the present invention provides a means firstly of ensuring that the amino acids or other coding groups are sufficiently ionised to give strong mass spectral peaks, and secondly of ensuring that each of the characteristic mass spectral fragments formed from the fragmentation of the coding sequence can be  
25 distinguished from extraneous peaks.

Accordingly, in another aspect, the invention provides a method of determining the identity of a substrate R linked to a solid support Q by mass spectrometric means; the solid support Q having a coding sequence attached thereto by means of a connecting group  $Y^a$   
30 having a cleavage site cleavable to release a fragment  $F^a$  from the solid support, the fragment  $F^a$  comprising the coding sequence and at least a portion of the connecting group  $Y^a$ , wherein (i) the chemical fragment  $F^a$  contains a sensitising group G which sensitises the chemical fragment  $F^a$  to mass spectroscopic analysis; and preferably

(ii) the fragment  $F^a$  contains a means for imparting a characteristic signature to the  
35 mass spectrum of the fragment;

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the coding sequence comprising a sequence of coding groups the nature and order of which is indicative of the identity of the substrate R;

the method comprising cleaving the connecting group  $Y^a$  so as to release the fragment  $F^a$  from the solid support; subjecting the fragment  $Y^a$  to mass spectrometry under conditions effective to bring about mass spectral fragmentation of the coding group and the formation of mass spectral fragment ions corresponding to the loss of one or more coding groups from the coding sequence, and thereafter correlating mass spectral peaks of the mass spectral fragment ions with the molecular ion of the fragment  $Y^a$  to identify the sequence of the individual coding groups

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In the methods and constructs of the invention, as an alternative, or in addition, to including a coding tag which is chemically different from the substrate, a proportion of the substrate itself can be bonded to the solid support in such a way that it is not cleaved from the support under the conditions used to remove the rest of the substrate from the support. The remaining substrate can thus function as an analytical label enabling identification by a method such as mass spectrometry. More particularly, a proportion of the total substrate R in the construct can be linked to the solid support by means of a connecting group  $Y^b$  having a cleavage site which is cleavable to release a fragment  $F^b$  from the solid support, the fragment  $F^b$  comprising the substrate and at least a portion of the connecting group  $Y^b$ ; the connecting group  $Y^b$  not being cleavable to release substrate R under conditions effective to cleave the second cleavage sites in the groups  $Y^1R$ ,  $Y^2R$  and  $Y^n$  [if present]) and wherein:

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(i) the chemical fragment  $F^b$  contains a sensitising group G which sensitises the chemical fragment  $F^a$  to instrumental, e.g. mass spectroscopic analysis and/or:

(ii) the fragment  $F^b$  contains a means for imparting a characteristic signature to the mass spectrum of the fragment.

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Thus, after liberation of the main proportion of the substrate from the constructs for biological testing, fragment  $F^b$  can be selectively cleaved from the solid support to yield sensitised substrate which is readily analysable by methods such as mass spectrometry. This could be done, for example, after biological assays had been carried out on the main proportion of the substrate, biological activity having been found, and it was required to determine the identity of the substrate. Alternatively, fragment  $F^b$  can be cleaved from the solid support without first removing the other substrate from the solid support, for example as part of a quality control (QC) procedure to test the quality of the products of the solid phase synthesis. In this latter procedure, fragment  $F^b$  can be cleaved from the solid support along

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with fragments Fr and F<sup>a</sup>, analysis of the fragments Fr, F<sup>a</sup> and F<sup>b</sup> providing a full picture of the outcome of the synthesis.

5 The cleavage site of groups Y<sup>a</sup> and Y<sup>b</sup> is preferably cleavable under conditions corresponding to those needed to cleave the first cleavage sites in the groups Y<sup>1</sup>R and Y<sup>2</sup>R. Thus, the product or substrate-containing fragments and the coding tag-containing fragment can be cleaved from the solid support at the same time thereby permitting them to be analysed (e.g. by mass spectrometry) simultaneously.

10 The chemical fragment F<sup>a</sup> preferably contains a sensitising group G which sensitises the chemical fragment F<sup>a</sup> to instrumental, e.g. mass spectroscopic analysis.

15 In a preferred aspect of the invention, the construct is characterised in that the sensitising group G is generated by cleavage at the first cleavage site(s) of the groups Y<sup>1</sup>, Y<sup>2</sup>, Y<sup>n</sup>, Y<sup>a</sup> or Y<sup>b</sup> respectively. In this preferred embodiment of the invention, a moiety containing the sensitising group G is formed or introduced at the first cleavage site(s) by cleavage of the "skeleton" of the construct, as distinct from cleavage of a side chain or mere removal of a protecting group.

20 The sensitising group G renders the fragment Fr, F<sup>a</sup> or F<sup>b</sup>, and hence the substrate R, coding tag or substrate-containing coding tag, more sensitive to analysis by a given analytical technique, and in particular a mass spectrometric technique. Thus the sensitising group can be a group which is readily ionisable under the conditions encountered in a mass spectrometer, and in particular electrospray mass spectrometry, to afford a strong signal.

25 The ionisable sensitising group serves to ensure that the fragment Fr, F<sup>a</sup> or F<sup>b</sup> is ionised sufficiently in the mass spectrometer to give a strong response. This overcomes a problem inherent in many molecules synthesised by solid phase methods where a suitable ionising group is not present and analysis by high throughput mass spectral techniques is problematical.

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35 The ionisable group can be for example a basic amino group or a carboxylate group but preferably it is a basic amino group. It will be appreciated that the term "basic amino group" as used herein refers in particular to an amino group which is readily protonated.

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The basic amino group can be a primary amino group, a secondary amino group, or a tertiary amino group. Where the basic amino group is a tertiary amino group, it can be for example, a cyclic amino group such as piperidino, piperazino, pyrrolidino, or morpholino, piperidino or piperazino (e.g. N-methylpiperazino) being presently preferred.

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In accordance with a preferred embodiment of the invention, a moiety containing the sensitising group G, such as a basic amino group, is formed or introduced at the cleavage site as the chemical fragment Fr, fragment F<sup>a</sup> or F<sup>b</sup> (hereinafter collectively referred to as the analytical fragment) is cleaved from the solid support. The advantage of forming the sensitising group G in this way is that it avoids the potential problem of a pre-existing or preformed sensitising group interfering with the chemistry of the construct.

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The atoms or functional group making up the sensitising group G can be present in a masked form in the construct before cleavage of the analytical fragment from the resin, the cleavage conditions merely serving to unmask the sensitising group G. Alternatively, the atoms or functional group making up or containing the sensitising group G can be introduced at the cleavage site during the cleavage reaction. For example, where the sensitising group is a basic amino group, the nitrogen atom of the amino group can be present in the construct before cleavage, or it can be introduced during the cleavage reaction.

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When the sensitising group G is introduced from an external source during the cleavage reaction, it can form part of a larger moiety. For example, the group G can be introduced as part of a group X-G wherein X is the residue of a nucleophile or electrophile, for example a nitrogen-based or sulphur-based nucleophile. Examples of groups X-G include groups of the formula G-Alk-Nuc wherein Alk is an alkylene group and Nuc is a nucleophile such as a secondary amino group (e.g. the secondary amino group in N-methylpiperazine) or a thiolate group.

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It is preferred that the analytical fragment contains a means for imparting a characteristic signature to the mass spectrum of the fragment. The signature can advantageously be provided by incorporating into the fragment a "peak splitting" isotopic label. The peak splitting isotopic label comprises at least one atom that exists in a number of stable isotopic forms. By isotopic labelling of one or more particular atoms in the analytical fragment such that a given atom is labelled with a mixture of isotopes, the mass spectra of the molecular ions will appear as a characteristic pattern, the precise pattern depending on the

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relative amounts of the individual isotopes. Thus, for example, if a given atom in the fragment Fr is labelled such that 50% of the atoms are of one isotopic form and 50% are of another isotopic form, the mass spectrum will show the molecular ion as a characteristic doublet in which the peaks are of approximately equal height.

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The purpose of the peak splitting atom(s) is to provide a characteristic pattern which will characterise any peak in the mass spectrum originating from the analytical fragment Fr and/or F<sup>a</sup>, and/or F<sup>b</sup> thereby distinguishing such peaks from those due to extraneous materials.

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The analytical fragments Fr, F<sup>a</sup> and F<sup>b</sup> can be labelled the same or differently but in one preferred embodiment, they are labelled differently so as to produce different characteristic signatures. In this way, mass spectral peaks due to the coding tag can be distinguished from mass spectral peaks from the fragments containing the substrate.

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Examples of atoms that can be used as isotopic peak splitting labels include <sup>1</sup>H/<sup>2</sup>H (D), <sup>79</sup>Br/<sup>81</sup>Br, <sup>12</sup>C/<sup>13</sup>C, <sup>14</sup>N/<sup>15</sup>N and <sup>16</sup>O/<sup>18</sup>O.

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The analytical fragments Fr, F<sup>b</sup> and F<sup>a</sup> can each contain a single peak splitting isotopic label or more than one such label. For example, the isotopic label can be a single bromine atom in which case the peak for the molecular ion of the analytical fragment liberated following cleavage from the solid support will appear as a doublet. By introducing a second or subsequent peak splitting label(s), a more complex peak pattern will be produced for the molecular ion.

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The isotopic peak splitting label(s) preferably is/are located between the first and second cleavage sites of the groups Y<sup>1</sup> and Y<sup>2</sup>.

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The first and second cleavage sites in the groups Y<sup>1</sup> can be defined by first and second linker groups L<sup>1</sup> and L<sup>2</sup>, the first and second cleavage sites in the group Y<sup>2</sup> can be defined by first and second linker groups L<sup>1</sup> and L<sup>3</sup>, and the cleavage site in the group Y<sup>a</sup> can be defined by a linker group L<sup>a</sup>, which preferably corresponds to L<sup>1</sup>, L<sup>2</sup> and L<sup>3</sup> being selectively and orthogonally cleavable. A "spacer group" A can be interposed between each pair of first and second linker groups, or between the linker group L<sup>a</sup> and the coding tag, the spacer group A typically containing an isotopic peak splitting label.

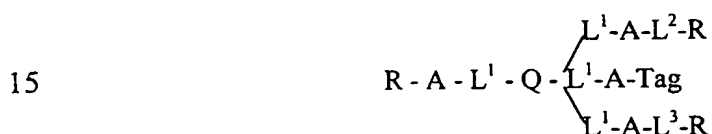
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Accordingly, one preferred embodiment of the invention can be represented by the formula:



wherein "Tag" represents a coding sequence.

In another preferred embodiment, the construct has a substrate-containing analytical group R-A linked to the solid support by a linker L<sup>1</sup> which is orthogonally cleavable with respect to the linkers L<sup>2</sup> and L<sup>3</sup>. A construct of this type can be represented by the formula:



In a further preferred embodiment, the coding sequence is omitted and the means of identifying the substrate is provided by the group R - A - L<sup>1</sup> which can be cleaved to provide sensitised substrate for analysis by mass spectrometry. Such a construct has the formula:



In another preferred embodiment, the invention provides a construct for use in a tiered release method of screening as described above, the construct containing a sensitised coding tag. Such a construct can be represented by the formula Tag - A - L<sup>1</sup> - Q - L<sup>1</sup> - A - L<sup>2</sup> - R.

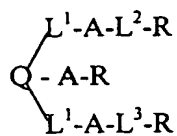
In other embodiments of the invention, the constructs can have a formula selected from the group consisting of:



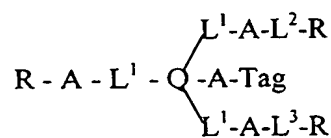
$$L^1-A-Tag$$

$$R-A-L^1-Q-L^1-A-L^2-R$$

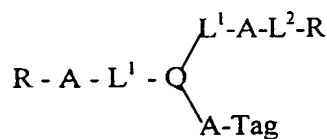
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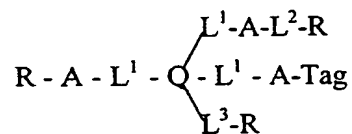
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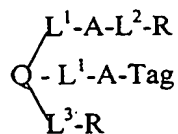
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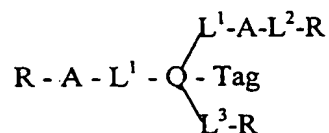
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$$R-L^3-Q-L^1-A-L^2-R$$


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wherein  $L^1$ ,  $L^2$ ,  $L^3$ , A and R are as hereinbefore defined and "Tag" represents a coding sequence.

The linkers  $L^1$ ,  $L^2$ , and  $L^3$  are orthogonally and selectively cleavable; i.e. the

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conditions used to effect cleavage at the cleavage site in one linker will not cleave the other. A wide variety of different types of cleavage reaction can be used, examples being reactions selected from acid catalysed cleavage, base catalysed cleavage, oxidative cleavage, reductive cleavage, nucleophilic displacement, electrophilic displacement, and thermal, photochemical and enzymatic cleavage.

Thus, in a preferred embodiment, there is provided a chemical construct as hereinbefore defined wherein the first cleavage site (e.g. as defined by the linker  $L^1$ ) is selectively cleavable by one type of chemistry selected from a group of chemistries consisting of cleavage under acid conditions, base catalysed cleavage, oxidative cleavage, reductive cleavage, nucleophilic displacement, electrophilic displacement, and thermal, photochemical and enzymatic cleavage, one of the second cleavage sites (e.g. as defined by the linker  $L^2$ ) is selectively cleavable by a second and different type of chemistry selected from the said group, and the other of the second cleavage sites (e.g. as defined by the linker  $L^3$ ) is selectively cleavable by a third and different type of chemistry selected from the said group.

Any one or more of the cleavage sites/linkers, can be of the "safety catch" variety; i.e. the cleavage site or linker group must be chemically modified in a first step before it can be subjected to cleavage in a second step. An advantage of such an arrangement is that it prevents or significantly reduces the possibility of cleavage taking place inadvertently. One example of a "safety catch" mechanism involves oxidation of a functional group in a first step, the oxidation serving to make the functional group more amenable to displacement by a nucleophile in a subsequent cleavage step. The nucleophile can vary considerably in structure. For example, in one embodiment, the nucleophile can be an amino group-containing nucleophile), the amino group participating in the nucleophilic displacement action such that the amino group is attached directly to the cleavage site. Alternatively, in another embodiment, the amino group (or other sensitising group) can be present in a group (e.g. a dialkylaminoalkyl-thiolate anion) containing another nucleophile such as a sulphur nucleophile which becomes attached to the cleavage site. In a further embodiment, the nucleophile can be a moiety containing a one basic nitrogen atom which functions as a nucleophile, and another basic nitrogen which functions as the sensitiser group G, an example of such a nucleophile being N-methylpiperazino.

Examples of linkers that can be selectively cleaved under acidic conditions include appropriately substituted benzyloxycarbonyl groups and substituted diphenyl-methylamino

groups, both of which can be cleaved by the action of trifluoroacetic acid.

Particular examples of acid cleavable linker groups are set forth in *The Combinatorial Index*, Barry A. Bunin, Academic Press, San Diego 1998, the disclosure of which is incorporated herein by reference. Linkers of the "Rink" or "Knorr" type typically comprise an N-protected 1-amino-1,1-diphenylmethane moiety, the amino group when deprotected allowing attachment to a substrate, one of the phenyl rings being substituted for example with dimethoxy groups and the other having a carboxyalkyloxy substituent providing a second point of attachment. Cleavage with trifluoroacetic acid gives rise to a substrate compound having a terminal carboxamido group. Linkers of the "Wang" type typically contain a substituted phenoxyacetyl group, the acetyl group providing one point of attachment, and a benzylic hydroxyl group on the phenyl ring forming a second point of attachment. Esters can be formed between a carboxyl group of a substrate and the benzylic hydroxyl group, the ester groups being subsequently cleavable with trifluoroacetic acid (TFA) to release a substrate compound having a terminal carboxylate group.

Examples of groups that can be selectively cleaved under photochemical conditions include carbamate groups such as *o*-nitrobenzyloxycarbamate groups in which initial cleavage takes place between the benzyloxy and carbonyl groups, elimination of carbon dioxide from the resulting fragment giving a basic amino group.

Examples of groups that can be cleaved by nucleophilic displacement include mercaptopyrimidine-based "safety catch" linkers such as 5-carboxy-2-mercaptopyrimidine, where cleavage can be effected by reacting under oxidising conditions to generate a sulphoxide or sulphone linkage, followed by reaction with a nucleophilic amino group to form a 2-aminopyrimidine. Particular examples of nucleophilic groups that can be used to effect cleavage of oxidised mercaptopyrimidine-based linkers include cyclic amines such as piperidine and N-substituted piperazine (e.g. N-methylpiperazine), and amino group-containing thiolate nucleophiles (e.g. dimethylaminoethylthiolate). Examples of oxidising conditions are those generated oxidising agents such as per-acids, e.g. a perbenzoic acid such as *m*-chloroperbenzoic acid, and certain inorganic per-salts such as potassium monoperoxysulphate.

By making the first and second cleavage sites, e.g. as defined by the first and second linker groups  $L^1$  and  $L^2$ , or  $L^1$  and  $L^3$  orthogonally cleavable, it is possible selectively to



separate from the construct either the analytical fragment or the substrate R, simply by using different cleavage conditions. This means that during experiments designed to optimise the conditions for a particular reaction step, the chemist can subject the construct to conditions suitable for cleaving off the analytical fragment thereby allowing analysis to be carried out to determine the outcome of each test reaction. Similarly, during a preparative scale reaction (e.g. a scale-up reaction or commercial production), quality control (QC) can be carried out by removing a number of solid supports from the reaction vessel, cleaving the constructs at the first cleavage site and analysing the resulting fragment to see whether a particular reaction step has worked. On the other hand, by cleaving at the second cleavage site e.g. on the second linker group, rather than the first, the reaction product R is released from the solid support. Thus, an advantage of the constructs of the present invention is that they can be used both at an experimental level to optimise a particular process step, and also at a preparative level without modifying the linker groups.

In one embodiment of the invention, the fragments Fr, F<sup>a</sup> or F<sup>b</sup>, and more preferably the spacer group A contains an alkylene diamine group or aminoalkoxy group. The precise size of the alkylene group and its degree of substitution is not currently considered to be important, but particular examples are ethylene or propylene diamine or aminoalcohol groups which may be substituted or unsubstituted. The alkylene diamine group or aminoalcohol typically contains a peak splitting isotopic label as hereinbefore defined. The two amino groups can each be bonded to respectively the first and second linker groups. In order to increase the mass of the spacer group A, or change its properties, the alkylene diamine group can be substituted by an aryl group, or example an N-aryl group such as an N-benzyl group, or the alkylene chain can be substituted (for example with one or more fluorine atoms to alter the volatility of the analytical fragment). The N-aryl group may optionally be substituted with one or more substituent groups.

Where, as is preferred, the spacer group contains one or more mass-spectral peak splitting isotopic labels, these can be located either in the alkylene chain or in a substituent group attached to the alkylene chain. Thus, for example, an N-benzyl group bonded to one of the two amino groups in an alkylendiamine can have a methylene group which is substituted with the peak splitting atom deuterium. Alternatively, an aryl ring (e.g. an N-benzyl group) present in a substituent on the alkylene diamine can be substituted with a peak splitting bromine atom, one particular example of an aryl group being an N-o-bromobenzyl group.

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The fragments Fr, F<sup>a</sup> or F<sup>b</sup>, and in particular the spacer group A, as well as providing a means of identification using mass spectrometry, can also be provided with one or more additional sensitisers to allow characterisation by other analytical techniques. For example, the spacer group can contain a chromophore to allow characterisation by ultra violet (u.v.) or fluorescence spectroscopy.

Although alkylene diamine and amino alcohol groups are given as specific examples of spacer groups, alternative spacer groups can be used. For example, the spacer groups can be formed from hydrocarbon chains containing up to thirty or more carbon atoms in the chain which can be optionally interrupted with one or more heteroatoms such as oxygen, nitrogen or sulphur. As a further alternative, the spacer can be for example a peptide chain containing one or more amino acids. The precise nature and length of the spacer is not currently considered to be important provided that the spacer does not interfere with the chemistry of the construct.

The solid support Q can be any type of solid support suitable for use in solid phase synthesis, and in particular combinatorial chemistry. Thus, purely by way of example, the solid support can take the form of beads, a solid surface, solid substrates, particles, pellets, discs, capillaries, hollow fibres, needles, solid fibres, or organic or inorganic gels such as silica gels, and insoluble organic particles such as particles formed from fullerenes.

Examples of beads are polymeric beads such as cellulose beads or resin beads, particular examples of materials from which resin beads can be prepared including functionalised polymer resins such as polystyrene resins, polyacrylamide resins and dimethylacrylamide resins. Examples of suitable supports are listed in *The Combinatorial Index* by Barry A. Bunin, referred to above.

In another aspect, the invention provides a method of analysing the constructs hereinbefore defined; the method comprising cleaving the groups Y<sup>1</sup> and Y<sup>2</sup>, and optionally the group Y<sup>a</sup> to release the chemical fragment Fr (and optionally the fragments F<sup>a</sup> and F<sup>b</sup> and then subjecting the chemical fragments Fr, and F<sup>a</sup> to mass spectrometry, e.g. electrospray mass spectrometry.

The analysis of the fragment Fr provides information on the reaction history of the construct. Thus, by mass spectrometric analysis, it can readily be determined whether or not

the desired substrate has been formed in a given reaction sequence. Analysis of the fragment Fr can therefore be used not only to characterise the substrate or product of the solid phase reaction sequence, but also to follow the progress of the reactions.

5 In a further aspect, the invention provides intermediate chemical constructs for use preparing a chemical construct as hereinbefore defined, the intermediate constructs having the formulae  $Y^{1'}-Q-Y^{2'}$ ,  $RY^{1'}-Q-Y^{2'}$  and  $Y^{1'}-Q-Y^{2'}R$  wherein  $Y^{1'}$  and  $Y^{2'}$  are reactive or protected form of the group Y; and R, Q and Y are as hereinbefore defined.

10 In a still further aspect, the invention provides intermediate constructs of the formulae  $L^{2'}-A-L^1-Q-L^1-A^p$ ,  $R-L^2-A-L^1-Q-L^1-A^p$ ,  $L^{3'}-A-L^1-Q-L^1-A^p$ ,  $R-L^3-A-L^1-Q-L^1-A-L^{2'}$ ,  $L^{3'}-A-L^1-Q-L^1-A-L^2-R$ , wherein  $L^{1'}$ ,  $L^{2'}$  and  $L^{3'}$  are reactive or protected forms of the linker groups  $L^1$ ,  $L^2$  and  $L^3$  hereinbefore defined,  $A^p$  is a reactive or protected form of the spacer group A containing a peak splitting isotopic label, and Q, R, A,  $L^1$ ,  $L^2$  and  $L^3$  are as hereinbefore defined. In a particular embodiment, the group  $A^p$  has the formula  $NH-Alk-NX^{1'}$  wherein Alk is an alkylene group and  $X^{1'}$  is hydrogen or an aralkyl group. The intermediate construct is preferably isotopically labelled with a peak splitting combination of atoms such as  $^1H/^2H$  (D),  $^{79}Br/^81Br$ ,  $^{12}C/^13C$ ,  $^{14}N/^15N$  and  $^{16}O/^18O$ .

20 In each of the aforementioned intermediates, the solid support can have optionally bonded thereto a coding tag sequence  $L^1-A-Tag$  and/or a substrate-based tag sequence  $R-A-L^1-$ , and precursor forms thereof such as  $L^1-A^p$ .

25 The invention will now be illustrated but not limited by reference to the following examples.

### **Brief Description of the Figures**

30 Figure 1 illustrates the mass spectrum of the product of the cleavage, by oxidation and nucleophilic displacement, of a construct of the formula  $Q-L^1-A-L^2-R$  wherein R is a model compound benzamide,  $L^1$  is a thiopyrimidine linker group, A is an N-bromobenzyl substituted ethylene diamine "peak splitting group" and  $L^2$  is a linker group which is cleavable under photochemical conditions.

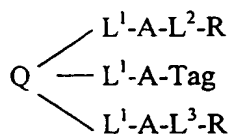
35 Figure 2 illustrates the mass spectrum of the construct of Figure 1 following

photochemical cleavage of the linker  $L^2$  and subsequent oxidation / nucleophilic displacement at linker  $L^1$ .

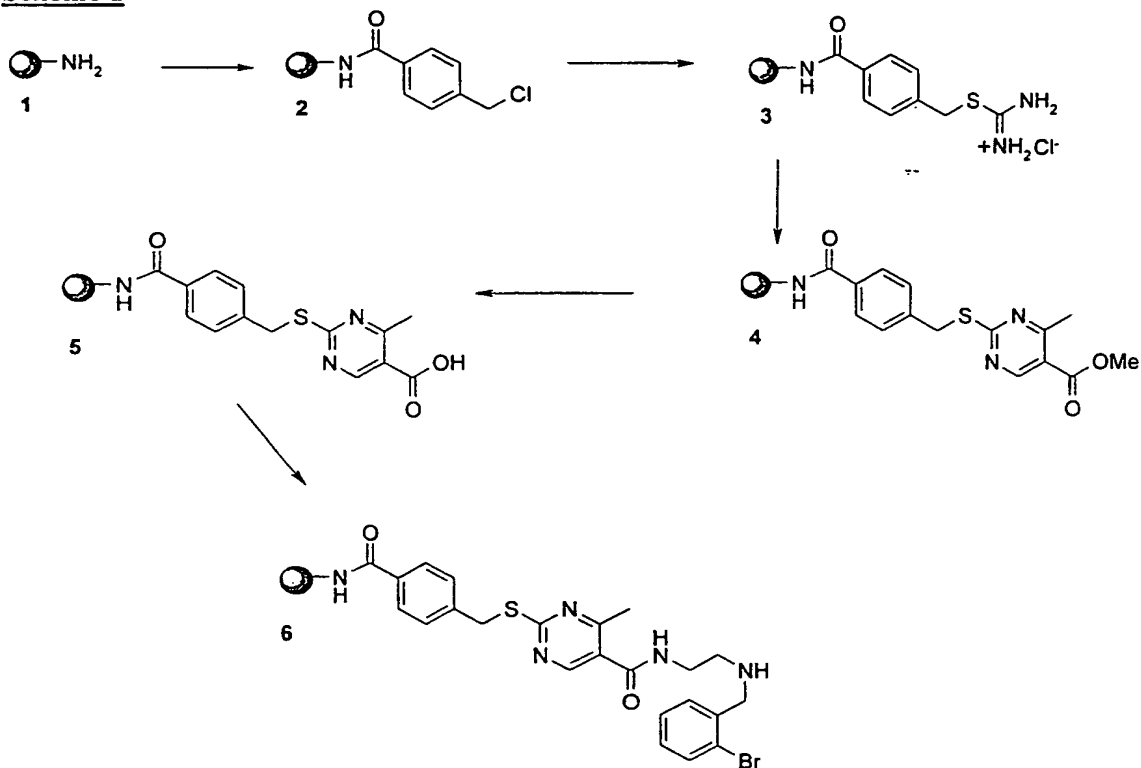
Figure 3 illustrates the mass spectrum of the product of the cleavage, by oxidation and nucleophilic displacement, of a construct of the formula  $Q-L^1-A-Tag$  wherein  $L^1$  is a thiopyrimidine linker group, A is the same peak splitting group as is present in the construct of Figure 1, and Tag is a coding sequence consisting of a tripeptide.

Figure 4 illustrates the mass spectrum of the cleavage product of Figure 3 but wherein the conditions within the mass spectrometer have been adjusted to cause fragmentation of the molecular ion at 716 a.m.u. in order to produce peaks due to constructs in which one or more amino acids have been lost from the molecular ion.

Figure 5 illustrates the mass spectrum of the product of the cleavage, by oxidation and nucleophilic displacement, of a construct of the formula:



wherein "Tag" represents an allyloxyglycine code group,  $L^1$ ,  $L^2$  and A are the same as for the constructs of Figures 1 to 4 and  $L^3$  is a linker of the "Rink" or "Knorr" type which is cleavable by trifluoroacetic acid.

**EXAMPLE 1****Scheme 1****Experimental for Scheme 1**

A solution of 4-(chloromethyl)benzoic acid (0.27 g, 1.6 mmol), Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)<sup>®</sup> (0.83 g, 1.6 mmol) and diisopropylethylamine (0.56 ml, 3.2 mmol) in dimethylformamide (8 ml) was shaken for 20 minutes and was then added to TentaGel  $\text{NH}_2$  (Rapp Polymere) resin 1 (1 g, 0.32 mmol). The resulting mixture was shaken for 5 hours, following which the resin was drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane and finally diethyl ether. The resin (2) was then dried *in vacuo*.

A slurry of thiourea (0.58 g, 7.7 mmol) in dioxane (4 ml) and ethanol (1 ml) was added to Resin 2 (1 g, 0.32 mmol) and the whole heated at 80 °C for 16 hours. The resin was drained and washed with dimethylformamide (x 5), dichloromethane (x 5), diethyl ether, dichloromethane and finally diethyl ether to give resin 3. The resin was then dried *in vacuo*.

A solution of methyl-2-(dimethylaminomethylene)-3-oxobutanoate (0.17 g, 0.96

mmol) and triethylamine (65 ml, 0.48 mmol) in dimethylformamide (10 ml) was added to resin 3 (1 g, 0.31 mmol) and the mixture was heated at 80 °C for 16 hours. The resin was then drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether. The washed resin (4) was dried *in vacuo*.

5

Resin 4 (0.5 g, 0.15 mmol) was then treated with a 1:1 mixture of tetrahydrofuran and 2N aqueous sodium hydroxide (4 ml) and shaken for 2 hours. The resin was then drained and washed with tetrahydrofuran/H<sub>2</sub>O, 10% acetic acid /tetrahydrofuran, tetrahydrofuran /H<sub>2</sub>O, tetrahydrofuran, dimethyl-formamide, dichloromethane, diethyl ether, dichloromethane, diethyl ether, and then dried *in vacuo* to give resin 5.

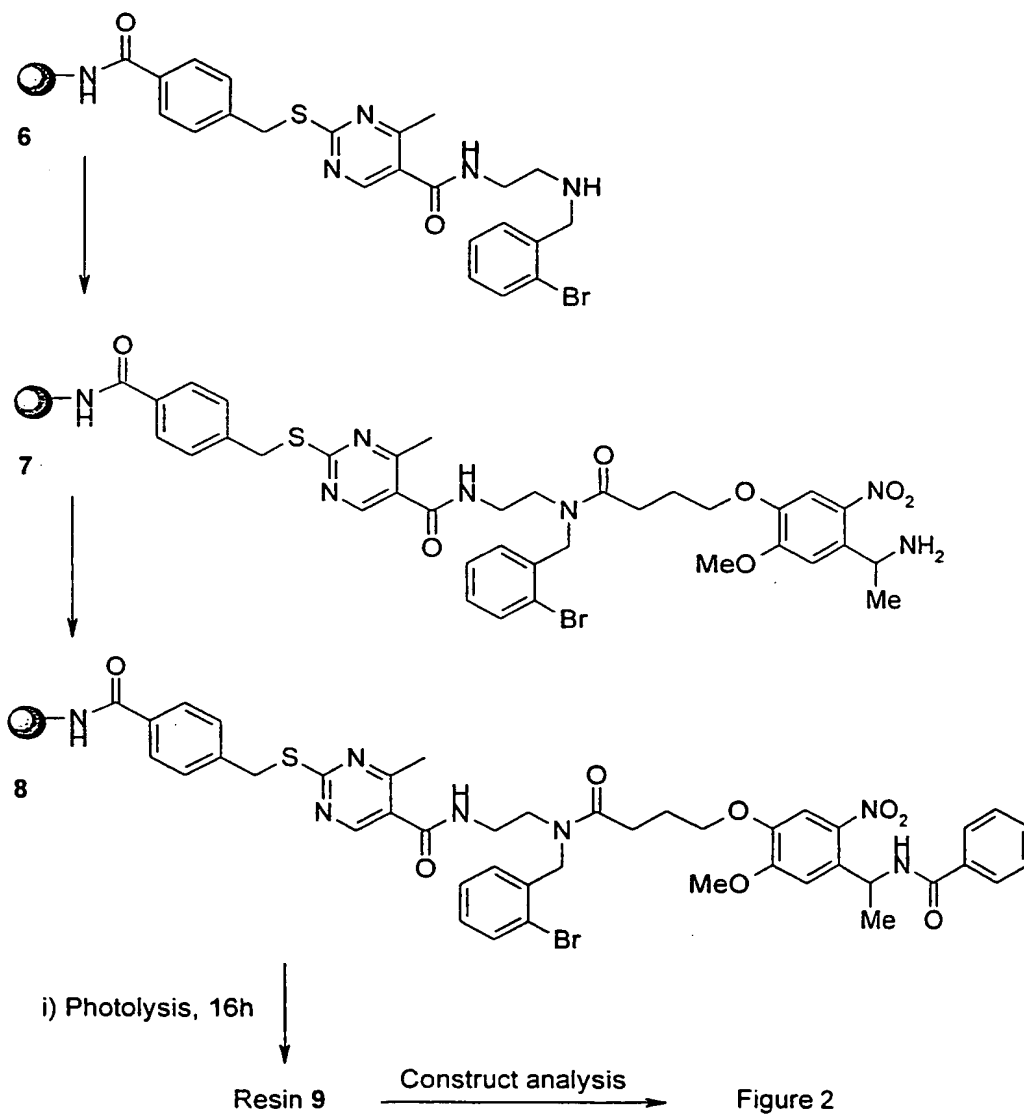
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A solution of PyBOP<sup>®</sup> (24 mg, 0.045 mmol) and diisopropylethylamine (16 ml, 0.09 mmol) in dimethylformamide (1 ml) was added to resin 5 (50 mg, 0.015 mmol) followed by a solution of 1-*tert*-butoxycarbonyl-1-(*o*-bromobenzyl)-diaminoethane (15 mg, 0.045 mmol) in dimethylformamide (1 ml) and the mixture was shaken for 3 days. The resin was drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether. A solution of 95% aqueous trifluoroacetic acid in dichloromethane (20%, 1 ml) was then added to the resin and the mixture was shaken for 2 hours. The resin was drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and then shaken with 10% diisopropylethylamine in dimethylformamide for 10 minutes. The resin was drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether. The resin (6) was then dried *in vacuo*.

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**EXAMPLE 2****Experiment to Demonstrate Residual Drug on Construct after photolysis**5 **Scheme 2**

### Experimental for Scheme 2

A solution of 4-[4-(1-(9-fluorenylmethoxycarbonylamino)ethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (23 mg, 0.045 mmol), diisopropylethylamine (16 ml, 0.09 mmol) and 2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (17 mg, 0.045 mmol) in dimethylformamide (2 ml) was added to resin 6 (50 mg, 0.015 mmol) and the whole was shaken for 3 hours. The resin was drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether. The resin was then treated with 20% piperidine in dimethylformamide (3 ml) and the whole was shaken for 1 hour. The resin (7) was then drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether and dried *in vacuo*.

A solution of benzoic acid (11 mg, 0.09 mmol), diisopropylethylamine (16 ml, 0.09 mmol) and 2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (17 mg, 0.045 mmol) in dimethylformamide (2 ml) was added to resin 7 and the mixture was shaken for 2 hours. The resin (8) was then drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether.

Analysis of the product of the synthesis was carried out by subjecting the resin-bound construct to thiopyrimidine cleavage conditions (described below), following which the construct was analysed by mass spectrometry. The mass spectrum of the construct is shown in figure 1.

A solution of 0.2% Hydrazine hydrate in dimethylsulphoxide (0.1 ml) was added to resin 9 (2 mg, 0.6 mmol) and the suspension subjected to photolysis for 16 hours. The photolysed resin (9) was then drained and washed with dimethylsulphoxide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether and dried *in vacuo*. The washed resin was subjected to pyrimidine cleavage conditions (described below) and the resulting cleavage product was analysed by mass spectrometry. The mass spectrum is shown in figure 2.

### General method of Construct Analysis of Resins via the Pyrimidine Linker.

A small sample of resin (ca. 0.5 mg) is treated with 0.01M m-chloroperbenzoic acid



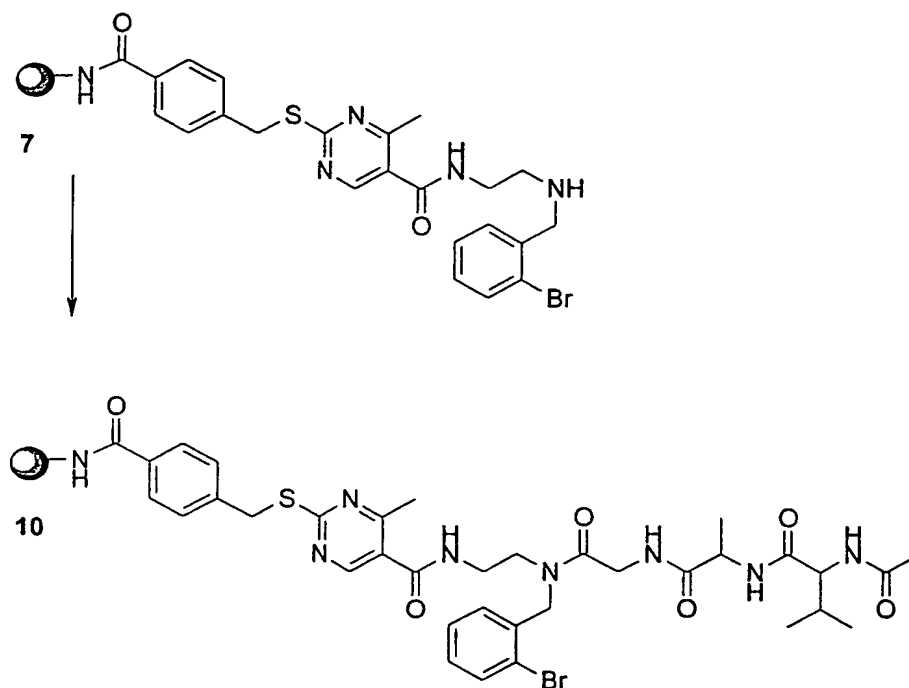
(0.5 ml) for 2 hours. The resin is then drained and washed with dichloromethane, diethyl ether and dichloromethane. The resin is then treated with 0.02 M N-methylpiperazine in dimethylformamide (50 ml) and the mixture is shaken for 12 hours. The solution is then removed and analysed by mass spectrometry.

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### EXAMPLE 3

#### Identification of a sensitised sequence of amino acids

##### 5 Scheme 3



#### Experimental for Scheme 3

10 Resin 10 was prepared by reacting resin 7 with the desired 9-fluorenylmethoxycarbonyl protected amino acids followed by deprotection, and then repeated coupling to the next amino acid and deprotection (see general method). The terminal amino group was then acylated using acetic anhydride (see method below).

15 The resulting resin was subjected to pyrimidine cleavage conditions (described above) followed by mass spectroscopic analysis. The mass spectrum is shown in Figure 3. Having identified the molecular ion at mass 716, the mass spectrum was run again at a higher voltage in order to bring about fragmentation and sequential cleavage of the three amino acids. The mass spectrum of the fragmented construct is shown in Figure 4.

#### General/Typical method for coupling amino acids to the linker

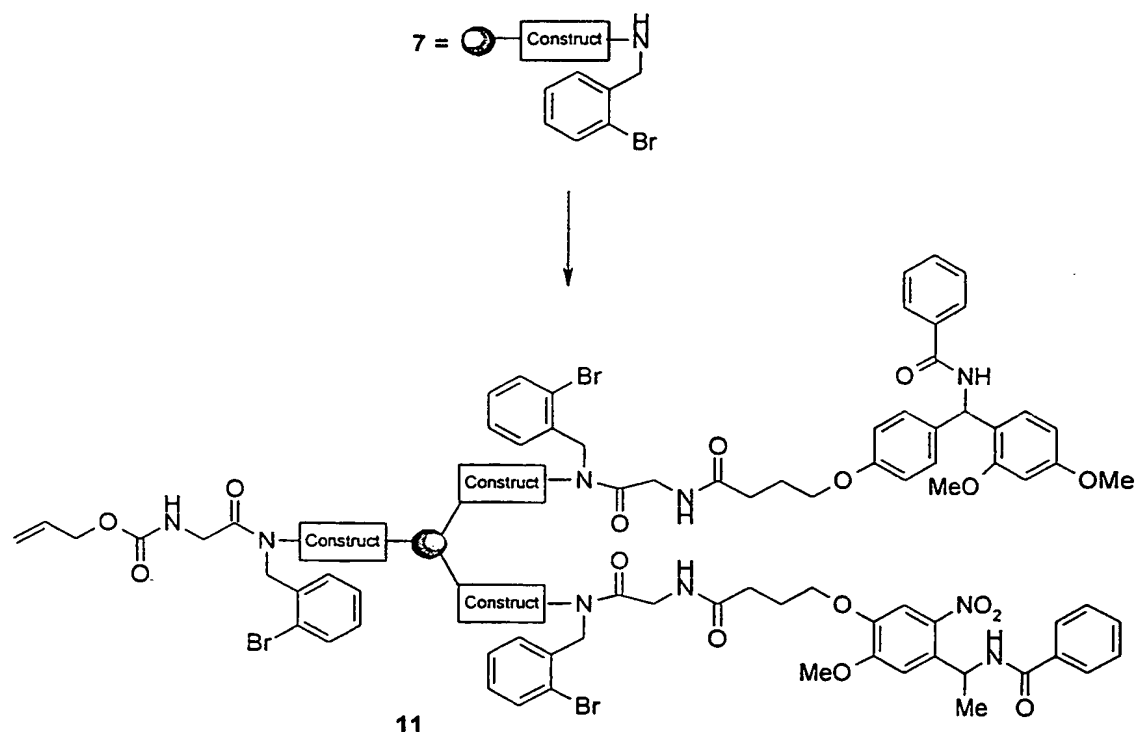
25 A solution of the 9-fluorenylmethoxycarbonyl protected amino acid (0.14 mmol, 10 Eq), diisopropylcarbodiimide (22 ml, 0.14 mmol, 10 Eq) and N-hydroxybenzotriazole (19 mg, 0.14 mmol, 10 Eq) in dimethylformamide (1ml) was added to required resin 7 (30 mg,

ca. 0.014 mmol). The mixture was then shaken for 5 hours. The resin was then drained and washed with dichloromethane, dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether. The 9-fluorenylmethoxycarbonyl protecting groups were then removed by the treatment of the resins with 20% piperidine solution in  
5 dimethylformamide (2 ml) and shaking for 30 minutes. The resin was then drained and washed with dichloromethane, dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether. The washed resin was dried *in vacuo*.

**Acetylation of the dipeptides**

10 A solution of acetic anhydride (15 ml, 0,14 mmol) in dichloromethane (1 ml) was added to the resins (30 mg, ca. 0.014 mmol) followed by 4-dimethylaminopyridine (1 mg, 0.008 mmol) and the mixture was shaken for 2 hours. The resin was then drained and washed with dichloromethane, dimethylformamide, dichloromethane, diethyl ether, dichloromethane,  
15 and finally diethyl ether. The washed resin was dried *in vacuo*.

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**EXAMPLE 4****Preparation of Differential Release Resin 11****5 Scheme 4****10 Experimental for Scheme 4**

Resin 7 (0.1 g, 0.03 mmol) was treated with a solution of 9-fluorenylmethoxycarbonyl glycine (36 mg, 0.12 mmol), N-allyloxycarbonyl glycine (5.2 mg, 0.03 mmol), PyBOP (78 mg, 0.15 mmol) and diisopropylethylamine (52 ml, 0.3 mmol) in dimethylformamide (1 ml) and the resulting mixture was shaken for 3 hours. The resin was then drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether and dried *in vacuo*.

The resin thus produced was treated with 20% piperidine in dimethylformamide (2 ml) and shaken for 30 minutes before draining and washing as described above. The resin was then treated with a solution of 4-[4-(1-(9-fluorenylmethoxycarbonylamino)ethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (39 mg, 0.075 mmol), p-[(R,S)- $\alpha$ -[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxy-benzyl]-phenoxybutanoic acid (43 mg, 0.075 mmol), PyBOP (78 mg, 0.15 mmol) and diisopropylethylamine (52 ml, 0.3 mmol) in

dimethylformamide (1ml) and the mixture was shaken for 3 hours. The resin was then drained and washed with dimethylformamide, dichloromethane, diethyl ether, dichloromethane, and finally diethyl ether and dried *in vacuo*.

- 5            This resin was then treated with 20% piperidine in dimethylformamide (2 ml) and shaken for 30 minutes. The resin was then drained and washed as above. The resin was then treated with a solution of benzoic acid (18 mg, 0.15 mmol), 2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (57 mg, 0.15 mmol) and diisopropylethylamine (52 ml, 0.3 mmol) in dimethylformamide (1 ml) and the whole was  
10            shaken for 2 hours. This resin was then drained and washed as above.

            The product resin was subjected to pyrimidine cleavage conditions (described above) followed by mass spectroscopic analysis, and the mass spectrum thus obtained is shown in figure 4.

15

            It will readily be apparent that numerous modifications and alterations could be made to the constructs described in the examples above without departing from the principles underlying this invention, and all such modifications and alterations are intended to be embraced by the claims appended hereto.

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